

# Fluorimetric study on the interaction between fluoresceinamine and bovine serum albumin\*

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Fluorescence spectroscopy was employed to investigate the interaction between fluorophore fluoresceinamine (FA) and bovine serum albumin (BSA) under physiological conditions. In the mechanism discussion, it was proved that the fluorescence quenching of BSA by FA is a result of the formation of a BSA-FA complex. Fluorescence quenching constants were determined using the modified Stern-Volmer equation to provide a measure of the binding affinity between FA and BSA. The results of the thermodynamic parameters  $\Delta G$ ,  $\Delta H$ , and  $\Delta S$  at different temperatures indicated that several kinds of interactions, except for the electrostatic interactions play cooperative roles in BSA-FA association. Furthermore, the conformation of BSA upon interaction with FA was also studied by synchrotron fluorescence spectroscopy.

Keywords: Bovine serum albumin, Fluoresceinamine, Fluorescence quenching, Binding constant, Protein conformation

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## I. INTRODUCTION

Fluorophore labeled proteins have been applied to *in vitro/vivo* research for decades [1–3]. Normally for robust labelling, chemical crosslinking has to be involved. For example, the well-known EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) crosslinking chemistry between the primary amines and the carboxylic groups [4, 5], as well as other specific chemical crosslinking pairs between the NHS ester and primary amines, thiol groups, and maleic acid imides (maleimides). Alternatively, the binding constant can also be high between some natural biological conjugating pairs such as enzyme-ligands, biotin-avidin, protein A/G-antibodies, antibody-antigens, aptamer-ligands, and complementary nucleic acids. Compared to chemical labeling, such biological binding pairs have been applied to separation/purification [6] and immobilization [7] for a long time. As a result, the physical binding is capable of recycling, due to the reversible binding, by altering the incubation conditions, such as pH, ionic strength, solvent polarity, and so on. Hence, developing a novel method to physically label proteins by fluorophores holds potent potential for biomedical applications.

In this paper, we have found that bovine serum albumin (BSA) can be stably labeled with fluoresceinamine (FA) via physical binding. To understand the strong interaction between the BSA and the FA molecules, the fluorescence quenching method and synchrotron fluorescence spectroscopy were employed to study the thermodynamic interaction parameters and the conformational changes of BSA, respectively.

## II. EXPERIMENTS

### A. Materials

BSA (lyophilized powder, > 98%), FA (> 95%), and all other chemical reagents were purchased from Sigma-Aldrich Corporation. The deionized water (18.2 MΩ cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA). BSA was dissolved in Milli-Q water for a stock solution with a concentration of 1 μM. FA was dissolved in a SB9 (sodium borate 50 mM, pH = 9) buffer with a stock concentration of 100 μM.

### B. Fluorescence measurements

Fluorescence spectra were recorded with a fluorescence spectrometer (Fluorolog®-MAX 4, Horiba) equipped with a 1.0 cm quartz cell. The excitation wavelength was fixed at 280 nm and the emission spectra were recorded at 300–550 nm. Both excitation and emission slits were set up to 5 nm. For the fluorimetric titration experiment, different solutions with a total volume of 0.9 mL containing different FA/BSA molar ratios with a fixed final BSA concentration of 0.33 μM (Table 1) were prepared and incubated at different temperatures (298, 304 and 310 K) for 30 min before measurements. The results were analyzed by using the (modified) Stern-Volmer equation.

TABLE 1. The molar ratios of FA/BSA for the fluorometric titration experiments

Vial number	1	2	3	4	5	6	7	8	9
BSA	1	1	1	1	1	1	1	1	0
FA	0	1.56	3.13	6.25	12.5	25	50	100	<sup>a</sup>

<sup>a</sup> Here FA's concentration is 33 μM.

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### C. UV-vis absorption spectroscopic measurements

UV-vis absorption spectra were recorded on a U-2900 UV-Vis spectrometer (Hitachi).

## III. RESULTS AND DISCUSSION

### A. Fluorescence characteristics of BSA-FA

Fluorescence quenching is a sensitive method used to investigate the interaction between a protein and molecules when the environment and the protein's structure are changed [8, 9]. The mechanism of fluorescence quenching is usually classified as either dynamic or static. Dynamic quenching normally results from diffusive encounters between the fluorophore and the quencher during the lifetime of the excited state, which is a time-dependent process. In contrast, static quenching occurs as a result of the formation of a non-fluorescent ground-state complex between the fluorophore and the quencher. Dynamic and static quenching can be distinguished by their dependence on temperature [10]. Because higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to rise with temperature. In contrast, the increasing temperature is likely to result in a decrease in the stability of complexes, thus lowering the values of the static quenching constants [1].

When BSA was excited at 280 nm it showed a strong fluorescence emission at 340 nm due to two types of amino acid residues, tyrosine and tryptophan. The effect of FA (structure shown in Fig. 1(a)) on the fluorescence spectra of BSA was explored and the changes are shown in Fig. 1(b). Upon addition of FA to the BSA solution, the fluorescence intensity showed a progressive decrease around 340 nm. The decrease in the fluorescence intensity of BSA can be observed regularly and acutely with an increasing concentration of FA, which confirms that FA quenches the intrinsic fluorescence of BSA and the quenching effects depend on the concentration of FA molecules. To clarify the fluorescence quenching, we performed fluorescence tests at different temperatures (298, 304 and 310 K), which can be described using the well-known Stern-Volmer equation (Eq. (1))

$$\frac{F_0}{F} = 1 + K_{\text{Sv}}[\text{FA}] = 1 + k_q \tau_0 [\text{FA}], \quad (1)$$

where,  $F_0$  and  $F$  are the fluorescence intensities in the absence and presence of the quencher (FA), respectively. The Stern-Volmer quenching constant is given by  $K_{\text{Sv}} = k_q \tau_0$ , where  $k_q$  is the quenching constant,  $\tau_0$  is the lifetime of the fluorophore in the absence of a quencher, and  $[\text{FA}]$  is the concentration of the quencher FA.

In this work, the concentration of the BSA solution was fixed at 0.33  $\mu\text{M}$  and the curve i in Fig. 1 is the emission spectrum of FA alone, which indicates that FA does not significantly emit in this wavelength range, thus can be negligible at the current excitation wavelength (280 nm). However, the standard Stern-Volmer plots (the inset of Fig. 1(b))

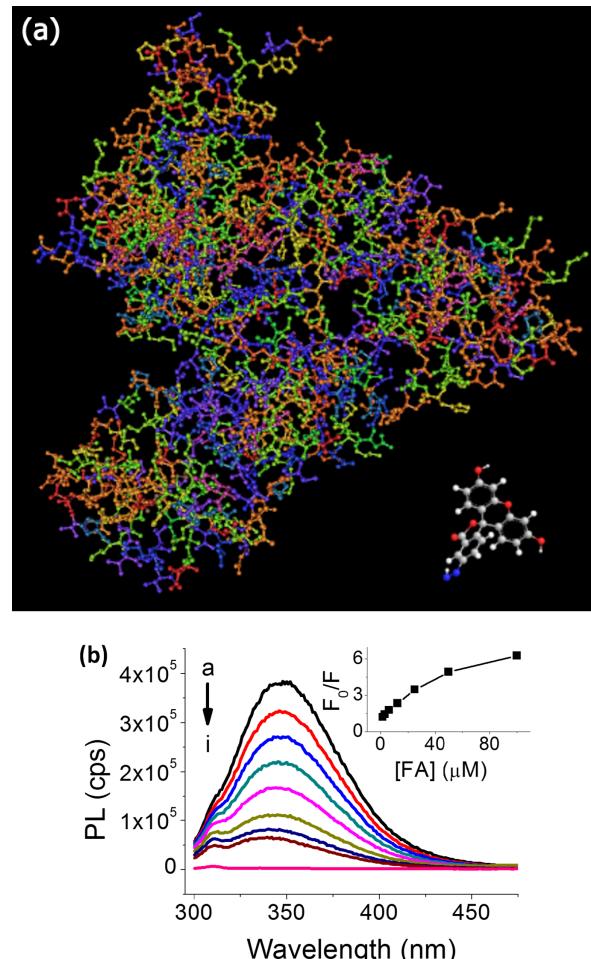


Fig. 1. (Color online) (a) Stick-Ball model of BSA and FA molecules. (b) Emission spectra of BSA in the presence of various concentrations of FA. The photoluminescence (PL) intensity was measured in counts per second (cps).  $c(\text{BSA}) = 0.33 \mu\text{M}$ . The molar ratios of  $c(\text{FA})/c(\text{BSA})$  from a to h are indicated in Table 1 (vial number 1–9). The curve i shows the emission spectrum of FA ( $c = 33 \mu\text{M}$ ) alone. The inset corresponds to the Stern-Volmer plot. ( $T = 298 \text{ K}$ ,  $\lambda_{\text{ex}} = 280 \text{ nm}$ ).

show a downward curvature (concave towards  $x$ -axis), indicating that the chromophoric residues are not all accessible to the quencher molecules, which might be caused by the static quenching due to the BSA-FA complex formation [10].

### B. Binding parameters

In this case, the PL data was further examined using the modified Stern-Volmer equation (Eq. (2))

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a} \frac{1}{[\text{FA}]} + \frac{1}{f_a}, \quad (2)$$

where,  $f_a$  is the fraction of accessible fluorophores and  $K_a$  is the effective quenching constant for the accessible fluorophores, which are analogous to the associative

TABLE 2. Binding constants and relative thermodynamic parameters of BSA-FA interaction

T (K)	Modified Stern-Volmer equation				$\Delta H^0$ (kJ/mol)	$\Delta G^0$ (kJ/mol)	$\Delta S^0$ (kJ/(mol K))
	$K_a$ (M)	$f_a$	$R$	$SD$			
298	$1.33 \times 10^5$	0.94	0.995	0.06	-96.88	-29.53	-0.23
304	$7.04 \times 10^4$	0.96	0.997	0.08	—	-28.17	—
310	$5.37 \times 10^4$	0.97	0.999	0.07	—	-26.82	—

coefficient of the quencher-acceptor system. The dependence of  $F_0/(F_0 - F)$  on the reciprocal value of the quencher concentration,  $[FA]^{-1}$ , should be linear with the slope of  $(f_a K_a)^{-1}$ , whereas the value  $f_a^{-1}$  is fixed on the ordinate. Therefore, the constant  $K_a$  is a quotient of  $f_a^{-1}$  and the slope  $(f_a K_a)^{-1}$ .

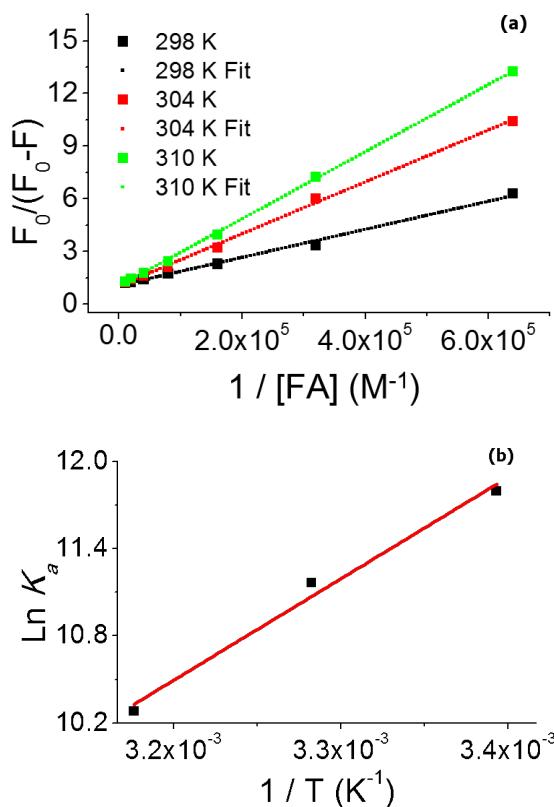


Fig. 2. (Color online) (a) The modified Stern-Volmer plot (dots) of BSA and the corresponding linear fit (lines) at different temperatures (298, 304 and 310 K), as indicated.  $F$ ,  $F_0$ , and  $[FA]$  represent the fluorescence/PL intensities of BSA in the presence or absence of the quencher (FA) and the concentration of FA, respectively. (b) Van't Hoff plot (black dots) at three different temperatures (298, 304 and 310 K) and the corresponding linear fit (red line).

The corresponding results shown in Fig. 2(a) at different temperatures are listed in Table 2. The decreasing trend of binding constants with increasing temperature indicates that BSA-FA adsorption is initiated by the conjugate/complex formation (static quenching). The results summarized in Table 2 also show that the binding constant between FA and BSA is moderate and the effect of temperature can not be neglected,

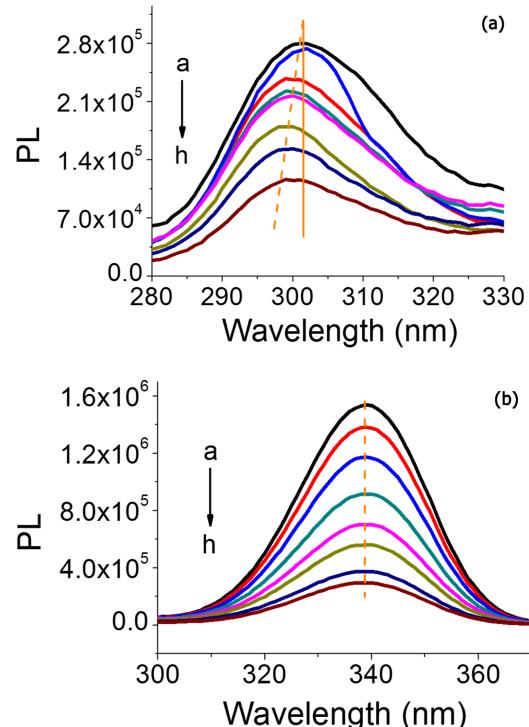


Fig. 3. (Color online) The synchronous fluorescence spectra of BSA at 15 nm (a) and 60 nm (b) wavelength interval. The molar ratios of  $c(FA)/c(BSA)$  from a to h are same as the series (from vial 1–8) shown in Table 1, respectively, and the  $c(BSA)$  was fixed at  $0.33 \mu\text{M}$  ( $T = 298 \text{ K}$ ).

in that the interaction between BSA and FA can be accelerated by increasing temperature. Hence, the stability of FA labeled BSA should be considered when handled at higher temperatures.

### C. Thermodynamic parameters

All chemical, physical, and biological processes are accompanied by a change in thermodynamic parameters ( $\Delta G^0$ ,  $\Delta H^0$ , and  $\Delta S^0$ ), which can be further studied by employing the van't Hoff equation

$$\ln K_a = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R}, \quad (3)$$

where  $K_a$  is the binding/association constant and  $R$ ,  $T$ ,  $\Delta H^0$ , and  $\Delta S^0$  are the gas constant (8.314 J/(mol K)), absolute tem-

perature, standard enthalpy, and standard entropy, respectively.

By plotting “ $\ln K_a$ ” against “ $1/T$ ”, as shown in Fig. 2(b), both values of  $\Delta H^0$  and  $\Delta S^0$  can be determined from the slope and intercept, respectively. The value of free energy change,  $\Delta G^0$ , can be determined by the Gibbs-Helmholtz equation

$$\Delta G^0 = \Delta H^0 - T\Delta S^0. \quad (4)$$

The interaction processes of the current study were found to be spontaneous, as evidenced by negative  $\Delta G^0$  values (Table 2). On the other hand, both the negative  $\Delta H^0$  ( $-96.88 \text{ kJ/mol}$ ) and the positive  $\Delta S^0$  ( $-0.23 \text{ kJ/(mol K)}$ ) values indicated that the BSA-FA interaction can be spontaneous because the physical adsorption of FA-BSA releases heat to environment/surroundings and, in fact, the  $\Delta S^0$  of the whole system, including the surroundings and the FA-BSA reaction system should be positive, which is consistent with the second law of thermodynamics.

In addition, the sign and magnitude of the thermodynamic parameters for protein reactions are usually used to identify the main forces contributing to ligand-protein stability [11]. Normally, the interactions between molecules and proteins can be classified into 5 catalogues: 1) hydrophobic interactions, 2) hydrogen mediated interactions, 3) coulombic/electrostatic interactions, 4) van der Waals forces, and 5)  $\pi$ - $\pi$  stacking interactions. According to the thermodynamic study of protein association reactions [12], in the case of  $\Delta H^0 < 0$  and  $\Delta S^0 < 0$ , only the coulombic/electrostatic interactions were ruled out (both FA and BSA in a pH 9 buffer are negatively charged by measuring their zeta potentials) and all the other interactions could be involved in the formation of BSA-FA conjugates/complexes.

#### D. Confirmation investigation

Spectroscopy is an ideal tool to observe conformational changes in proteins, since it allows for non-intrusive measurements of substances in a low concentration under physiological conditions. Synchronous fluorescence spectroscopy

(SFS) is a multidimensional fluorescence technique, which has become an increasingly active subject of research ever since its introduction by Lloyd [13]. The constant wavelength SFS technique involves simultaneous scanning of the excitation and emission monochromators, while maintaining a constant wavelength interval between them. The SFS gives information about the molecular environment in the vicinity of the chromophore molecules [14]. When the  $D$ -value ( $\Delta\lambda$ ) between the excitation wavelength and emission wavelength is stabilized at 15 nm or 60 nm, the synchronous fluorescence gives the characteristic information of tyrosine residues or tryptophan residues [15].

The effect of FA on BSA synchronous fluorescence spectroscopy is shown in Fig. 3. It is apparent from Fig. 3 that the maximum emission wavelength blue shifts (from 302 to 299 nm) at the investigated concentration range when  $\Delta\lambda = 15 \text{ nm}$  (Fig. 3(a)) and the maximum emission wavelength blue shifts (from 340 to 338 nm) when  $\Delta\lambda = 60 \text{ nm}$  (Fig. 3(b)). The blue shift of the emission maximum suggests a less polar (or more hydrophobic) environment of chromophore (tyrosine and tryptophan) residues in BSA.

#### IV. CONCLUSION

In this paper, the interaction between FA and BSA was studied by spectroscopic methods. The experimental results indicate that the interaction between BSA and FA involves hydrophobic,  $\pi$ - $\pi$  stacking, van der Waals forces and hydrogen-bond mediated interactions. The static quenching mechanism can be used to understand that there is BSA-FA complex formed spontaneously during the interaction. Furthermore, the protein's conformation was also studied by SFS. The biological significance of this work is that the BSA-FA conjugates formed merely by physical adsorption might apply to bio-labeling and reversible bio-sensors.

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